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UNDERSTANDING HOW LAND USE CHARACTERISTICS AFFECT THE PREVALENCE OF ANTIBIOTIC RESISTANT, VIRULENT *E. COLI* AND HOST-SPECIFIC MARKERS IN WATERSHEDS WITH AND WITHOUT SWINE CAFOS

By
Elizabeth Christenson and Jill Stewart

Department of Environmental Sciences and Engineering
University of North Carolina at Chapel Hill
Chapel Hill, NC

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Introduction

The US Environmental Protection Agency (EPA) cites pathogens as the largest cause of surface water impairment, and EPA attributes the most probable source to agriculture from field runoff (*1*). In confined animal feeding operations (CAFOs) housing swine, large volumes of manure are typically stored in deep, open-air lagoons and subsequently sprayed onto sprayfields as fertilizer for crops. Without proper nutrient management and manure application procedures, nutrients and pathogens can leach into groundwater or be transported to surface water as runoff.

North Carolina (NC) is the second largest producer of swine in the United States (*2*) and CAFOs have been identified as a nonpoint source pollutant for nutrients in the surface water of our state (*3, 4*). Nutrient management and responsible application of manure is necessary to prevent surface water runoff or groundwater infiltration (*2*). Previous work from the USGS (*5*) compared small NC watersheds with CAFOs and those without CAFOs and identified "measurable effects of CAFO waste manures on stream water quality" by measuring nutrient content and also found that some land use characteristics such as a higher density of wetlands had a protective effect against nutrient contamination. However, this study did not measure the effect of CAFO presence in the watershed on water quality with respect to pathogens, source of pathogens, or antimicrobial resistance.

Although NC Department of Environmental Quality (DEQ) regulates nutrients from CAFOs they do not regulate microbial transport. Chronic microbial contamination has been identified in southeastern NC related directly to swine CAFOs (*4*). Testing surface water for fecal indicator bacteria, such as *E. coli*, is commonly used as an indicator for fecal pollution and human pathogen exposure (*6, 7*). Enterohemorrhagic *E. coli* (EHEC) are virulent strains of *E. coli* in humans with a low infectious dose that can cause severe gastrointestinal illness and has caused waterborne outbreaks (*2*). EHEC can originate in swine and one study found viable EHEC in surface water after a large swine manure spill (*8*). Additionally, a recently developed technique, microbial source tracking (MST), enables source identification of fecal contamination by targeting a gene "marker" specific to the host source (e.g. swine, chicken, or human). MST can help determine whether fecal contamination detected in water originated from swine or a different source (see, *e.g.*, (*9–11*)). Additionally, droplet digital polymerase chain reaction (ddPCR) can be used to quantify the MST markers rather than testing only their presence or absence. ddPCR is a novel technology that splits a sample into many droplets and identifies the presence or absence of the target gene in each droplet (*12*). The output is a proportion of positive target gene reads. ddPCR has fewer technical barriers than standard curve-based real time PCR (qPCR) for quantification, as it is not as easily affected by inhibition, making it especially useful for environmental samples (*12, 13*). In NC, although swine-specific MST markers have been validated in swine manure (*14*), and although the transport of pathogens from swine CAFOs to surface water is documented, no studies have compared the presence of pathogenic *E. coli* and host-specific MST markers between watersheds with different land use relating to industrial swine production.

Finally, in addition to pathogen transport from livestock operations, antibiotic resistance can be passed to human pathogens (*15*) and so the transport of antibiotic resistant (ABR) bacteria from CAFOs is of concern (*16*). Current agricultural practices commonly administer antibiotics to

livestock to treat and prevent disease, and an estimated 75% of antibiotics are not absorbed but pass directly into the waste (*16*).

This study systematically compares watersheds with different land use characteristics, primarily with respect to swine CAFOs, and their effect on microbial water quality. The hypothesis was that antibiotic resistant *E. coli*, virulent *E. coli*, and swine-specific MST markers would be higher in watersheds with swine CAFOs compared to similarly sized agricultural watersheds without any CAFOs. The specific research objectives of this project were to (1) quantify microbial pollution as defined by *E. coli* concentration, (2) determine antibiotic resistance of *E. coli*, (3) determine virulence of *E. coli*, and (4) quantify microbial source tracking (MST) indicators to identify sources of *E. coli* between watersheds with and without swine CAFOs in NC.

Methods

Surface waters were collected and tested for selected microbial endpoints over the course of a year in watersheds with and without swine CAFOS. Sample selection was based on an earlier USGS study (*5*) that compared nutrient contamination at these same sites. In total, nine background sites and thirteen swine sites were sampled up to nine times between August 2016 and August 2017 for a total of 196 sampling events. Background sites were defined as having an upstream watershed land area that is primarily agricultural land and does not contain any type of CAFO or wastewater treatment plant. Swine sites were defined as having an upstream watershed land area that is primarily agricultural land and contains a swine CAFO barn and/or lagoon and/or sprayfield and does not have any other kind of CAFO or wastewater treatment plant. Approximately one liter of water was collected at each sampling site and sampling time and transferred on ice to the laboratory at the University of North Carolina at Chapel Hill. Samples were processed at the laboratory within 24 hours of sample collection.

E. coli culture and quantification

Standard membrane filtration methods were used to quantify concentrations of *E. coli* from each water sample collected (*17*). Briefly, 50mL, 25mL, 5mL, and 1mL volumes of water were filtered through four membranes and the membranes were aseptically placed on plates containing selective mTEC media. The plates are inverted and incubated at 37^{o}C for 2 hr followed by 44 ^{o}C for 22 hr (+/- 2 hr) then colonies with morphological characteristics of *E. coli* were summed and used to calculate concentrations of colony forming units (CFUs) per 100mL. CFU was determined by averaging the CFU among countable dilutions. Up to five *E. coli* colonies per sample were then isolated, purified, confirmed through biochemical testing including indole production, and archived for further analysis.

Antimicrobial resistance testing

Antimicrobial resistance testing was conducted on all archived *E. coli* isolates using standard Kirby-Bauer disc diffusion methods and following standard CLSI guidelines (*18*). Isolates were tested for resistance to eleven antibiotics comprising nine antibiotic classes as recommended by NARMS (*19*) and CLSI (*18*) guidelines. Tested antibiotics included antibiotics used primarily in industrial agriculture (*20*) and antibiotics used primarily in human medicine (*21*) with risk assessment priority levels assigned based on WHO criteria (*22*) (Table 1). Multi-drug resistance was defined as resistance to three or more antibiotic classes. Isolates were also screened for

carbapenem resistance, AmpC β-lactamase production, and extended spectrum β-lactamase (ESBL) production, which are resistance traits of high public health concern. For this study, a positive screen for carbapenem resistance was resistance to imipenem, for AmpC β-lactamase production was resistance to cefoxitin, and for ESBL production was intermediate or complete resistance to ceftriaxone. Isolates with a positive screen for AmpC β-lactamase production were confirmed through the disc approximation test (*23*) while isolates with a positive screen for ESBL production were confirmed using CLSI protocol (*18*).

Table 1: Antibiotics included in antimicrobial resistance testing of *E. coli*

Antibiotic	Antibiotic Class	Veterinary Use	Human Use	WHO Priority
Amoxicillin-Clavulanate Acid	Penicillin	Yes	Yes	High Priority Critical
Ampicillin	Penicillin	Yes	Yes	High Priority Critical
Cefoxitin	Cephalosporin	No	Yes	Highly Important
Ceftriaxone	Cephalosporin III	No	Yes	Highest Priority Critical
Chloramphenicol	Amphenicol	Yes	Yes	Highly Important
Ciprofloxacin	Fluoroquinolones	No	Yes	Highest Priority Critical
Gentamicin	Aminoglycosides	Yes	Yes	High Priority Critical
Imipenem	Carbapenem	No	Yes	High Priority Critical
Levofloxacin	Fluoroquinolones	No	Yes	Highest Priority Critical
Tetracycline	Tetracyclines	Yes	Yes	Highly Important
Trimethoprim-Sulfamethoxazole	Sulfas	No	Yes	Highly Important

Virulence testing

E. coli isolates were prioritized for virulence characterization if they were multi-drug resistant or had a positive screen for carbapenem, AmpC, or ESBL production. Virulence testing was conducted at the NCSU Clinical Microbiology Laboratory under the direction of Dr. Megan Jacob. Prioritized *E. coli* isolates were characterized using a multiplex polymerase chain reaction (PCR) assay for six virulence genes (*stx1, stx2, hlyA, rfbe, eae, flyC*) associated with the human pathogen *E. coli* O157:H7 (*24*) as well as two genes, *CMY2* and *TEM*, associated with ESBL production (*25*).

Microbial source tracking

During sample processing, 100mL of each sample were filtered through 0.45um polycarbonate filters and saved in MoBio PowerSoil DNA extraction tubes (Qiagen Inc, Germantown, MD) at -80^{o}C until extraction. DNA extraction using the PowerSoil kit was then conducted following manufacturer's protocol with one addendum that tubes were bead beaten for two minutes prior to extraction. Extracted DNA was then stored frozen at -80^{o}C until ddPCR analysis which was conducted as a duplex assay targeting pig2bac (*14*, *26*), associated with swine fecal contamination, and *Bacteroides* HF183, associated with human fecal contamination (*11*, *27*). Prior to running samples, the duplex assays were optimized for reaction temperature and assessed for assay competition. All samples (n=196) were then run in duplicate for pig2bac and HF183 using the optimized parameters.

To quantify the MST targets, the range of values for a negative droplet was determined by finding the mean amplitude of droplets in negative samples (e.g. negative extraction controls, negative template controls, and field blanks) plus or minus two standard deviations. The range of values for a positive droplet was determined from sample standards by subtracting out mean negative concentration and finding the mean amplitude of positive droplets plus or minus two standard deviations. The 95% confidence interval lower bound of this range was considered the threshold value to determine positive droplets in samples. In samples, a droplet was considered positive if above the threshold value for the gene target.

Results

E. coli culture and quantification

Of 196 total sampling events, 187 events were included in the quantitative, CFU analysis of *E. coli*. Four samples were not included in the CFU analysis because the site was dry at the time of sample collection, and five samples were excluded due to a laboratory error in culturing *E. coli*. Table 2 identifies the number of times a site was sampled, the number of times a CFU was determined, and the total number of confirmed *E. coli* isolates that were archived for each site. Sites were sampled an average of 8.5 times each between August 2016 and August 2017 with an average of 40 *E. coli* isolates archived for each background site and 43 *E. coli* isolates archived for each swine site.

Table 2. Sampling summary for 22 sites between August 2016 and August 2017 including the number of sites sampled, number of sampling events per site, number of times CFU was determined, and number of *E. coli* isolates archived.

Site	n sample dates	n CFU determined	n *E. coli* isolates
BK01U	9	9	47
BK03	9	9	45
BK05U	9	9	40
BK10U	9	8	24
BK12	9	9	38
BK14	9	9	43
BK15	9	9	43
BK16	9	9	41
BK17U	9	6	35
SW01	9	9	46
SW04	9	9	46
SW05	9	9	43
SW05A	9	8	43
SW05C	9	9	41
SW07	9	9	44
SW09	8	8	39

Site	n sample dates	n CFU determined	n *E. coli* isolates
SW10	9	9	45
SW11	9	9	45
SW13	8	8	40
SW16	9	8	43
SW17	9	8	39
SW17U	9	7	42

In total, 912 *E. coli* isolated from swine and background sites were confirmed as indole producers and have been archived. Figure 1 displays a boxplot of *E. coli* concentrations identified at all sites. The y-axis in Figure 1 is cut at 9000 CFU/100mL although a few concentrations are above this value. Additionally, among sampling events, 42% (n=77) of background sites and 70% (n=110) swine sites were above the EPA recommendation of 126 CFU/100mL for recreational waters.

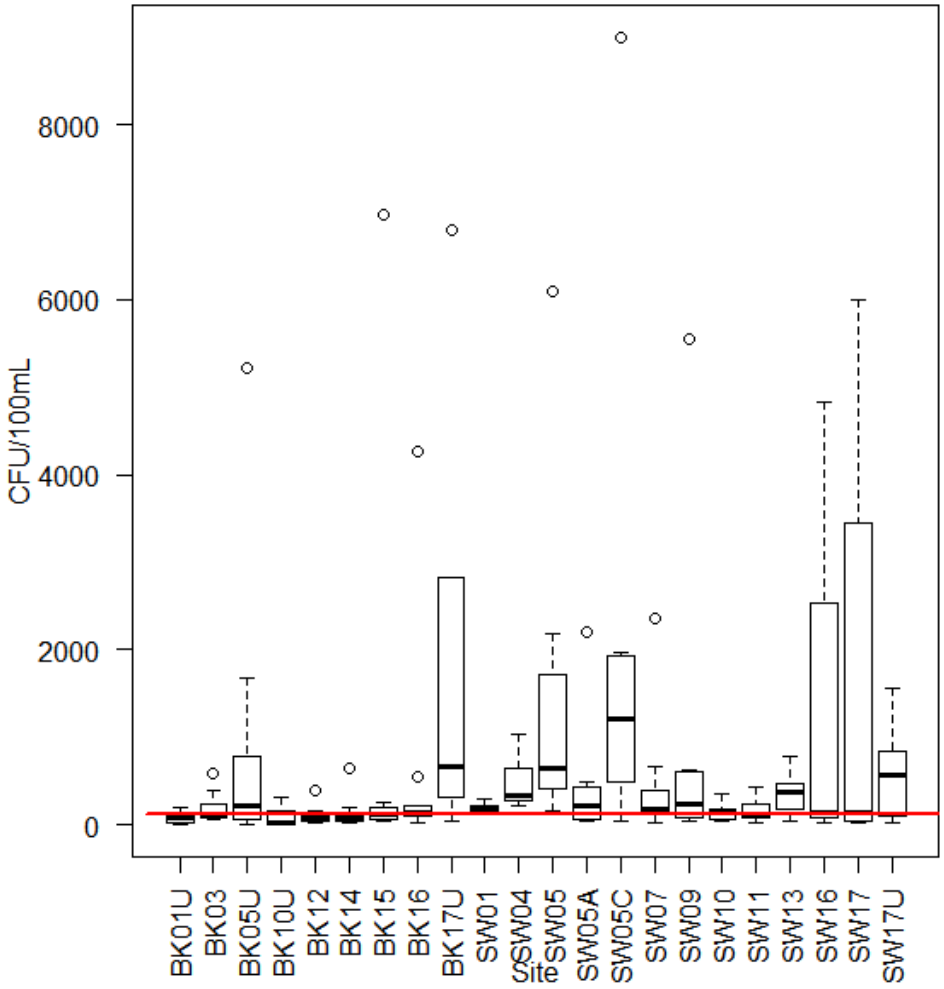

Figure 1: *E. coli* concentrations at all sites throughout sampling period. The red line represents the 2012 EPA recommendation for recreational waters of 126 CFU/100mL**.**

Table 3 identifies mean, minimum, and maximum *E. coli* concentrations for each site. Mean concentrations of *E. coli* in background sites was 501 CFU/100mL (95% confidence interval (CI)= 203-800) compared to mean concentrations of 1188 CFU/100mL (CI= 522-1854) in swine sites.

Table 3: Concentrations of *E. coli* observed for each site including background (BK) and swine (SW) sites.

Site	Average (CFU/100mL)	95% Standard Error (CFU/100mL)	Minimum (CFU/100mL)	Maximum (CFU/100mL)
BK01U	89	48	2	200
BK03	202	116	56	580
BK05U	937	1106	10	5220
BK10U	83	70	0	306
BK12	99	78	12	392
BK14	145	127	18	640
BK15	880	1493	40	6970
BK16	625	896	26	4260
BK17U	1885	2089	42	6800
SW01	193	34	124	292
SW04	3719	6441	220	30000
SW05	1485	1215	154	6100
SW05A	462	504	44	2210
SW05C	1874	1807	43	9000
SW07	483	475	20	2350
SW09	922	1303	40	5540
SW10	155	81	35	358
SW11	160	88	20	432
SW13	357	159	47	780
SW16	2714	3945	22	16200
SW17	2308	2865	12	11200
SW17U	571	416	24	1550

Antimicrobial resistance testing

Of 912 *E. coli* isolates archived, 356 background and 556 swine *E. coli* isolates were tested for resistance to eleven antibiotics including screening for carbapenem resistance, AmpC β-lactamase production, and ESBL production. Table 4 displays the results of antimicrobial resistance testing. Antimicrobial resistance to at least one antibiotic was observed in isolates collected from swine sites (19%, n=556) more often than background sites (6%, n=356) (Table 4,

Figure 2)**.** For every antibiotic with observed resistance, resistance was more often observed in isolates from swine sites compared to those from background sites. Tetracycline resistance was the most commonly observed with 17% of swine isolates compared to 5% of background isolates followed by ampicillin resistance in 5% swine isolates compared to 0.8% background isolates.

Table 4: Number and percent of *E. coli* isolates with observed resistance to antibiotics from water samples collected from background and swine sites. Observed resistance does not include observed intermediate resistance. Amoxicillin-clauvanate acid (AmC), ampicillin (AM), ceftriaxone (CRO), chloramphenicol (C), ciprofloxacin (CIP), cefoxitin (FOX), gentamycin (GM), imipenem (IPM), levofloxacin (LVX), sulfamethoxazole-trimethoprim (SXT), and tetracycline (TE).

Sample Type	AmC	AM	CRO	C	CIP	FOX	GM	IPM	LVX	SXT	TE
Background	1 (0.3%)	3 (0.8%)	0	0	0	1 (0.3%)	0	0	0	0	19 (5.4%)
Swine	4 (7%)	28 (5%)	7 (1.3%)	5 (0.9%)	2 (0.4%)	4 (0.7%)	2 (0.4%)	0	2 (0.4%)	7 (1.3%)	96 (17%)

Figure 2 identifies the percent of isolates from swine and background sites and the number of antibiotic classes with observed resistance. Swine sites were more likely to be resistant to a higher number of antibiotic classes. Multi-drug resistance, defined as resistance to three or more classes of antibiotics, has been observed among 2.5% (n=556) of *E. coli* isolates from sites downstream from swine CAFOs, and from 0.28% (n=356) of *E. coli* from background sites (Figure 3). Multi-drug resistance was observed in one isolate from a background site compared to 14 isolates from four swine sites across twelve sampling events.

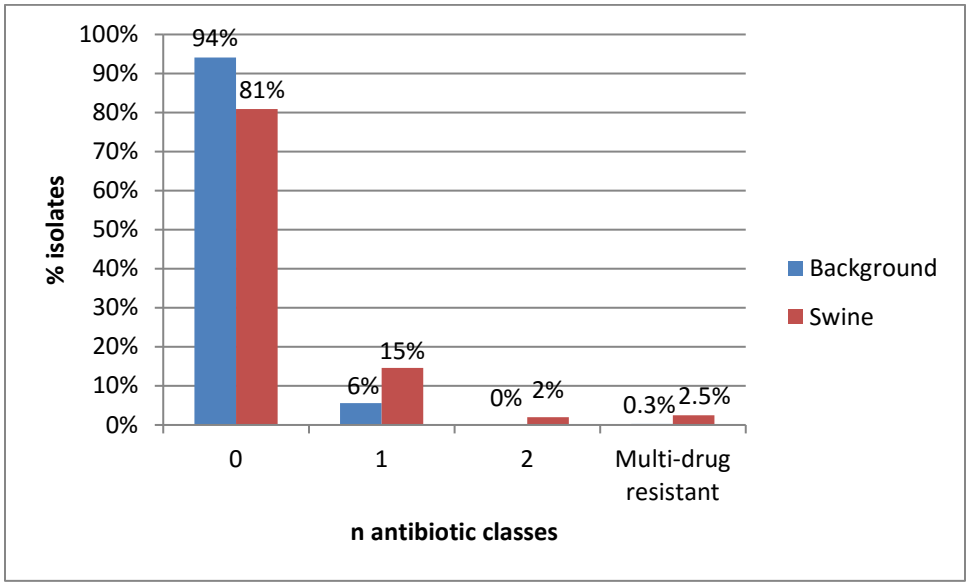

Figure 2: The percent of isolates from each observational group that are resistant to 0, 1, 2, or more (i.e. multi-drug resistant) classes of antibiotics.

Screens for carbapenem resistance, AmpC β-lactamase production, and ESBL production were conducted for all archived isolates (n=912). Results are summarized in Table 5. No isolates had a positive screen for carbapenem production since imipenem resistance was not observed. One isolate was confirmed as AmpC-producing and four isolates were confirmed as ESBL-producing. Confirmed AmpC and ESBL-producing isolates (n=5) were from swine sites.

Table 5: Results of multi-drug resistance, screening, and confirmation tests for carbapenem, AmpC, and ESBL production among *E. coli* isolates.

Sample Type	Multi-Drug Resistant isolates (n)	Positive Screen (n)			Positive Confirmation (n)	
		Carbapenem	AmpC	ESBL	AmpC	ESBL
Background	1	0	0	1	0	0
Swine	14	0	1	8	1	4

Virulence testing

Virulence testing was conducted on prioritized *E. coli* isolates (n=17) comprising all isolates with a positive screen for AmpC production (n=1) or ESBL production (n=9), and all isolates that were multi-drug resistant (n=15). Two isolates were tested that were not multi-drug resistant but had a positive screen for AmpC or ESBL production. An additional swine isolate remains to be tested for a total of 18 isolates that will be characterized. Of isolates prioritized for virulence testing, 16 were from swine sites and one was from a background site (Figure 3).

Isolates tested did not carry both ESBL genes *TEM* and *CMY2*, but rather 13 of the 17 isolates (76%) were positive for one of the two ESBL targets. Additionally, isolates with a positive confirmation of ESBL by culture did not have either ESBL gene target. One isolate from a site downstream of swine CAFOs was also positive for *stx2*, encoding shiga toxin production, and *hlyA*, encoding hemolysin toxin production (Table 6). These results show that virulence factors can be detected in waterborne *E. coli* isolates near swine CAFOs.

Table 6: Characterization of multi-drug resistant *E. coli* isolates from swine and background sites for extended-spectrum beta-lactamase (ESBL) production and genes associated with the human pathogen O157:H7.

Sample Type		ESBL		O157:H7					
	n tested	*TEM*	*CMY2*	*stx1*	*stx2*	*hlyA*	*rfbe*	*eae*	*flyC*
Background	1	0	1	0	0	0	0	0	0
Swine	16	8	4	0	1	1	0	0	0

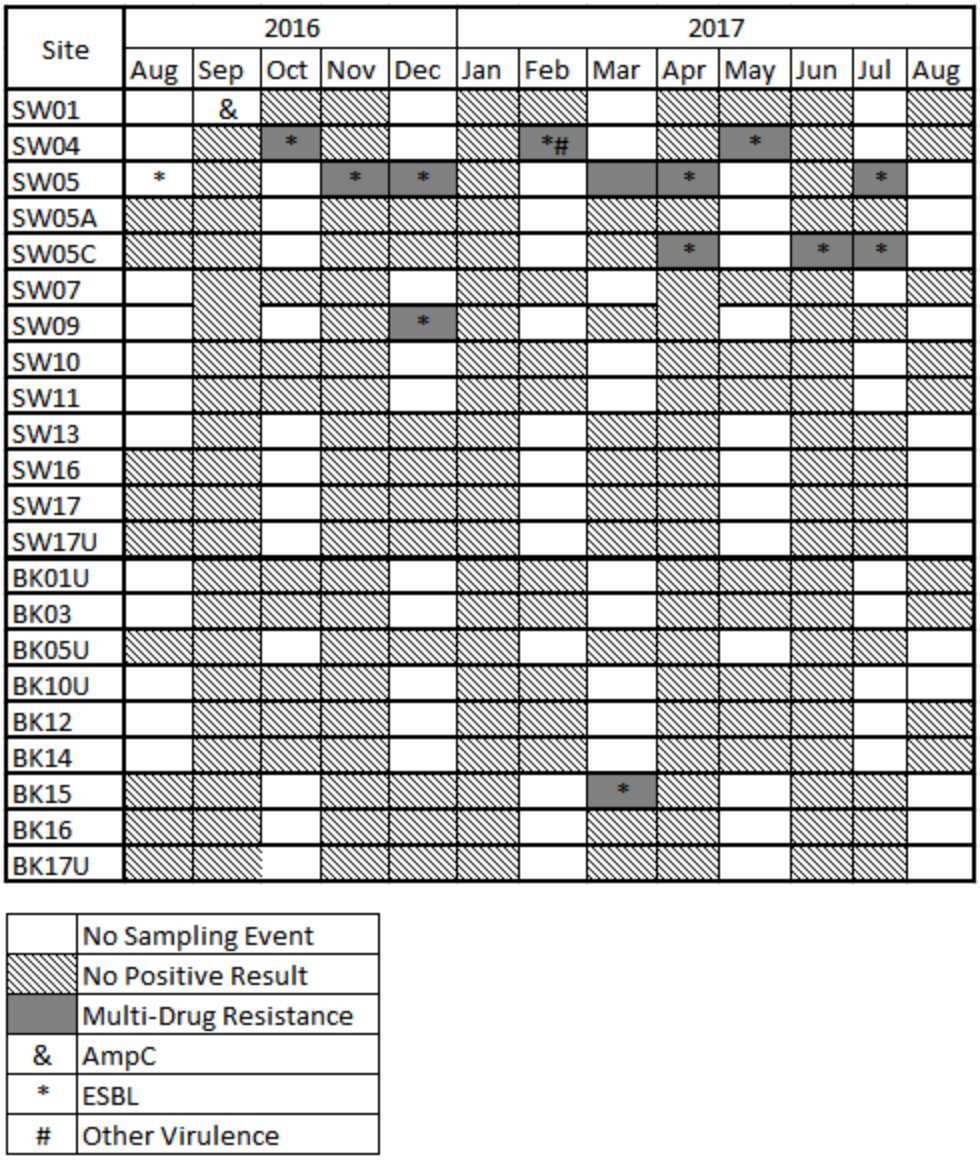

Figure 3: Sites and associated sampling events with at least one isolate positive for multi-drug resistance (grey) and/or virulence characteristics (AmpC - &, ESBL - *, and other - #). ESBL positivity is considered positive by culture or one of two genetic targets, and other virulence (#) indicates positivity for at least one of six virulence factors that characterize O157:H7.

Microbial source tracking

All samples (n=194) were run in duplicate for pig2bac and HF183 using the duplex ddPCR assay and data analysis is ongoing. Resulting gene target concentrations are subject to quality control steps such as ensuring that droplet generation is over 10,000 droplets and ensuring threshold values are based on positive and negative control ranges.

Discussion

In our landscape-scale watershed study of the effects of land use on the presence of virulent, antibiotic-resistant *E. coli* and microbial source markers, we found higher mean concentrations of *E. coli* at swine sites compared to background sites (1188 CFU/100mL (CI= 522-1845) vs. 503 CFU/100mL(CI= 203-800)), higher antibiotic resistance to at least one antibiotic at swine compared to background sites (19% vs. 6%), higher multi-drug resistance at swine sites compared to background sites (2.5% vs. 0.28%), and higher number of virulence factors at swine sites compared to background sites. Additionally, among sampling events, 42% (n=77) of background sites and 70% (n=110) swine sites were above the EPA recommendation of 126 CFU/100mL for recreational waters.

Our future work is to finish assessment of ddPCR microbial source tracking gene targets HF183 and pig2bac among samples and to use exploratory research techniques to assess potential relationships of spatial covariates with microbial outcomes. With variables of interest collected, we are now able to begin modeling efforts to identify the impacts of environmental variables including precipitation, distance to nearest sprayfield, percent wetland in watershed, steady state live weight of hogs permitted upstream of sampling point, and human population from census block data. Previous work has identified that geospatial data can be used in combination with water quality data to identify the source of nonpoint source pollution (*5*, *6*). Land use characteristics, such as wetlands and vegetative river buffers, may help mitigate the effects of swine CAFOs on receiving surface waters. Wetlands have been shown to reduce pathogen and antimicrobial input into receiving surface waters from CAFOs (*28*, *29*). Our future work will analyze continuous variables such as percent wetland together with discretely collected variables such as *E. coli* concentration in water samples and CAFO-specific manure application data from state permits.

Training:

This fellowship provided research funding for PhD student Elizabeth Christenson to collect the data presented which will form the basis of her dissertation. Implementation of this project also included training of five undergraduate students and two master's level students who assisted with field and laboratory work including sample collection, media preparation, membrane filtration, *E. coli* culture and isolation, and antibiotic resistance testing. One master's student leveraged water samples collected from this field project to assess antibiotic resistant *Staphylococcus aureus* for a subset of the swine and background samples collected, analysis that will form the basis of her MS thesis. This fellowship also provided funding for one PhD student and one master's level student to attend the annual WRRI conference in Raleigh, NC in March 2017.

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Pooja Naik (BSPH, in progress)
Maddy Grace Ponder (BSPH, in progress)
Maggie Lucas (BS, in progress)
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*using data collected from this project as basis for thesis or dissertation

Presentations:

Christenson, E., Stewart, J. Prevalence of antibiotic-resistant *E. coli* in North Carolina watersheds with and without swine CAFOs. UNC Water Microbiology Conference. Chapel Hill, NC. May 15-17, 2017. [poster]

Stewart, JR. Tracking pathogens in coastal waters. North Carolina Coastal Conference. Raleigh, NC. April 5, 2017.

Christenson, E., Stewart, J. Prevalence of antibiotic-resistant *E. coli* in North Carolina watersheds with and without swine CAFOs. Water Resources Research Institute annual conference. Raleigh, NC. March 16, 2017.

Stewart, JR. Water research at UNC: Assisting communities across NC. NC Clean Tech Summit. March 3. Chapel Hill, NC. March 3, 2017.

Christenson, E. All that glimmers is not gold: Understanding how land use characteristics affect the prevalence of antibiotic resistant *E. coli* in watersheds with and without swine CAFOs. Environmental Sciences and Engineering department seminar. Chapel Hill, NC. March 1, 2017.

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